Br. J. Nutr. (1978), 40, 235

The level and origin of amylase (*EC* 3.2.1.1) in the digestive tract of chicks receiving trypsin inhibitors in their diet*

BY ZAFRIRA NITSAN AND Z. MADAR

Division of Poultry Science, Agricultural Research Organization, The Volcani Center and Department of Agricultural Biochemistry, Faculty of Agriculture, Hebrew University of Jerusalem, Rehovot, Israel

(*Received* 21 *September* 1977 – *Accepted* 17 *February* 1978)

1. Amylase (*EC* 3.2.1.1) activity found in the intestinal tract of chicks posterior to the stomach is of endogenous origin, as amylase in the food is inactivated by the low pH in the stomachs.

2. Ingestion of raw soya-bean diet (RSD) or of heated soya-bean diet (HSD) supplemented with trypsin inhibitors induced higher amylase activities in the lower part of the small intestine and caecum as compared with HSD.

3. Ingestion of RSD after ligation at the end of the duodenum, end of the ileum or one of the caeca, or injection of soya-bean trypsin inhibitor into a ligated caecum, indicated that there is no amylase synthesis by the intestinal tract cells or microflora as a response to the presence of RSD or trypsin inhibitors.

4. It seems that amylase found in the digestive tract of the chick is of pancreatic origin and that RSD or trypsin inhibitors induce higher pancreatic amylase secretion than HSD which (the additional amylase) accumulates mainly in the caeca.

It is well established that a raw-soya-bean diet (RSD), or trypsin (*EC* 3.4.4.4) and chymotrypsin (*EC* 3.4.4.5) inhibitors isolated from soya beans induce pancreas enlargement and increase synthesis of its enzymes in rats and chicks (Liener & Kakade, 1969; Gertler & Nitsan, 1970; Nitsan & Liener, 1976). It has been shown in rats that humoral factors are involved in this stimulation (Kayambashi & Lyman, 1966) and that a negative feedback mechanism controls the proteolytic enzymes levels in the pancreas and in the digestive tract (Green & Lyman, 1972). Trypsin and chymotrypsin inhibitors produce enzyme-inhibitor complexes in the intestine. This inhibition, which reduced the level of free trypsin and chymotrypsin, stimulated an increase in the secretion of pancreatic proteases (Green & Lyman, 1972). However, amylase (*EC* 3.2.1.1) is not inhibited by soya-bean inhibitors. There is contradictory evidence regarding the effect of RSD on the levels of amylase in the pancreas of chicks given RSD as compared with heated-soya-bean diet (HSD) (Lepkovsky, Koike, Sugiura, Dimick & Furuta, 1966; Nitsan & Gertler, 1972; Nitsan & Bruckental, 1977).

In the present work the levels of amylase in the different segments of the digestive tract, as affected by RSD or trypsin inhibitors, were determined. There was also an attempt to locate the origin of the high levels of amylase found in the caeca of chicks given RSD.

EXPERIMENTAL

Diets

Heated soya-bean meal (HSM) (Shemen Ltd, Haifa, Israel) was a commercial product containing 440 g protein (nitrogen × 6·25)/kg.

Raw soya-bean meal (RSM) was prepared by milling commercial soya-bean flakes (from the same source as the HSM), defatted with light petroleum (b.p. 40–60°), after evaporation of the solvent at room temperature.

* Contribution No. 214-E, 1977 Series, from the Agricultural Research Organization, The Volcani Center, Bet Dagan, Israel.

The compositions of the heated and the raw-soya-bean diets (HSD and RSD) were as described by Gertler & Nitsan (1970). Bowman-Birk trypsin inhibitor (BBTI) was prepared according to Birk & Gertler (1968).

Turkey ovomucoid (TOM) was prepared according to the procedure of Feeney, Osuga & Maeda (1967).

Determination of enzyme activities

Trypsin and chymotrypsin activities were measured using *N*-benzoyl-DL-arginine-*p*-nitroanilide hydrochloride and *N*-acetyl-L-tyrosine-*p*-nitroanilide (E. Merck, Darmstadt, W. Germany) as respective substrates (Erlanger, Kokowsky & Cohen, 1961; Erlanger, Edel & Cooper, 1966).

Amylase activity was determined by the method of Bernfeld (1955) with modifications described by Gertler & Nitsan (1970). Preparation of intestinal and caecal contents and activation of pancreatic and duodenal homogenates with enterokinase (*EC* 3.4.4.8) was described earlier (Gertler & Nitsan, 1970).

Enzymic activities were expressed in all instances as units of activity where one unit was defined as a change in extinction of one extinction unit at 410 nm/60 min for trypsin and chymotrypsin and at 550 nm/3 min for amylase, using 12·7 mm test-tubes (Bausch & Lomb, Rochester, NY 14602).

Experimental procedure

1-d-old male New Hampshire × White Leghorn chicks were given *ad lib.* a commercial diet containing 240 g protein/kg (Nir, Shapira, Nitsan & Dror, 1974) for 21 d. Food, but not water, was removed 18 h before the start of each experiment except in Expt 1.

Expt 1. *Amylase activity along the intestinal tract of chicks.* Twenty male chicks were given HSD or RSD for 21 d, at which age they were killed using a guillotine. After autopsy, the intestinal tract was removed, the contents of each segment were collected separately and weighed, and amylase activity was determined.

Expt 2. *The effect of pH upon soya-bean amylase activity.* Amylase was extracted by stirring a suspension of HSM or RSM in water (1:20, w/v) for 1 h. After centrifugation at 18000 rev./min, amylase activity was determined in the supernatant fraction which had a pH of 6·3 and after decreasing the pH with hydrochloric acid to 3·5 and 2·0.

Expt 3. *Effect of diet on amylase activity.* Forty-eight chicks were divided into eight groups according to body-weight. The chicks were tube-fed 5 g HSD or RSD, HSM or RSM, HSD supplemented with BBTI or TOM (4 mg/g diet), BBTI dissolved in saline (9 g sodium chloride/l) (20 mg/2 ml), and saline alone (2 ml) as a control. Tube-feeding was used to introduce equal amounts of food in a limited period of time to all chicks. The diets or soya-bean meals were mixed with lukewarm water (37°) (1:5, w/v) before feeding. One end of the tube was inserted into the crop while the other was connected to a peristaltic pump which introduced the food into the crop at a constant velocity of 3 ml/min. At 2 h after feeding, the chicks were killed, the pancreas, the contents of the small intestine and caecum were collected, weighed and frozen at −20° until amylase, trypsin, and chymotrypsin activities were determined.

Expt 4. *Study of the origin of the high levels of amylase found in the caecum after ingestion of RSD.* Chicks were anaesthetized with diethyl ether and the abdominal cavity was opened. In twelve chicks one caecum was ligated with cotton thread and then the incision was closed with 11 mm Michel wound clips (Empire Findings Co. Inc., 127 W. 24th St, New York, USA); the other caecum remained unligated and served as a control. In twelve chicks the end of ileum, 5 mm before the start of the caeca, was ligated; in twelve other chicks ligation was done at the end of the duodenum, 10 mm posterior to the entrance of the bile and pancreatic ducts. Approximately 1 h after operations the chicks were tube-fed with HSD,

Table 1. *Expt* 1. *Amylase* (EC 3.2.1.1) *activity along the digestive tract of chicks given heated* (*HSD*) *or raw* (*RSD*) *soya-bean diets* for* 21 *d*

(Mean values for ten chicks/group)

	HSD (units†/g chyme)	RSD (units†/g chyme)	SE of mean
Crop	2·2[b]	45·0[a]	3·00
Glandular stomach	7·5[a]	9·2[a]	0·83
Gizzard	1·3[a]	0·8[a]	0·17
Duodenum	85·0[a]	100[a]	11·1
Jejunum	350[a]	400[a]	25·2
Ileum	370[b]	970[a]	36·7
Caecum	80·0[b]	4200[a]	350

[a, b] Values followed by a common superscript letter did not differ significantly, ($P < 0.05$). Statistical analysis was carried out separately for each segment.

* For details, see p. 273.

† One unit of activity was defined as a change in extinction of one extinction unit at 550 nm/3 min.

Table 2. *Expt* 2. *Effect of pH on amylase* (EC 3.2.1.1) *activity* (*units*/g*) *in heated* (*HSM*)- *and raw* (*RSM*)-*soya-bean meals*†

pH	HSM	RSM
6·3	4·73	110
3·5	0·37	35·7
2·0	0·50	1·12

* One unit of activity was defined as a change in extinction of one extinction unit at 550 nm/3 min.

† For details see p. 273.

RSD or trypsin inhibtors dissolved in saline or mixed with HSD. After a further 2 h period, the chicks were killed and the small intestine and caecal contents were collected and frozen at −20° for further determination of amylase, trypsin and chymotrypsin. In twelve chicks the two caeca were ligated: to one caecum 5 mg BBTI dissolved in 1 ml saline was injected while the other caecum served as a control and was injected with 1 ml saline. The chicks were killed and treated (but not fed) as described previously.

The results were subjected to analysis of variance according to Snedecor (1962).

RESULTS

Expt 1. Amylase activity along the intestinal tract of chicks is shown in Table 1. Low levels of amylase were detected in the crop, glandular stomach and gizzard. Much higher levels were found in the segments of the small intestine, which decreased toward the distal end of the digestive tract in chicks fed HSD and significantly increased in the ileum and caeca of RSD-fed chicks.

Expt 2. The activity of soya-bean amylase was reduced at low pH (Table 2). It was found in vitro that approximately 90% of the residual amylase activity left in HSM (after the heat treatment) was inactivated when the pH was reduced from 6·3 to 3·5 or 2·0; for these pH reductions, the corresponding values for amylase activity in RSM were 70 and 99%.

Expt 3. As was found previously, ingestion of RSD caused an increase in the amylase activity in the caecum as compared with the levels found in HSD-fed chicks. However, RSM, when fed alone, did not induce higher amylase activity in the caecum than HSM (Table 3). HSD supplemented with BBTI or with TOM induced higher amylase activity in the caecum when compared with unsupplemented HSD. Higher amylase activity was found

Table 3. *Expt* 3. *Enzyme activities (units†/g chyme) in the small intestine and caecum of chicks given one meal of heated (HSD)- or raw (RSD)-soya-bean diet, heated (HSM)- or raw (RSM)-soya-bean meal or HSD plus trypsin inhibitor (BBTI) or turkey ovomucoid (TOM)**

(Mean values for six chicks/group)

	Small intestine		
	Amylase (*EC* 3.2.1.1)	Trypsin (*EC* 3.4.4.4)	Chymotrypsin (*EC* 3.4.4.5)
HSD	1218[a]	28·5[a]	10·0[a]
RSD	1792[a]	1·2[d]	1·3[e]
HSM	987[a]	18·3[a]	10·7[a]
RSM	851[a]	7·3[b]	0·5[e]
HSD+BBTI	1009[a]	0·9[e]	1·0[e]
HSD+TOM	2052[a]	3·6[e]	0·5[e]
Saline‡+BBTI	507[b]	3·5[e]	1·7[e]
Saline	763[b]	7·8[b]	3·1[b]
SE of mean	335	0·35	0·4

	Caecum		
	Amylase (*EC* 3.2.1.1)	Trypsin (*EC* 3.4.4.4)	Chymotrypsin (*EC* 3.4.4.5)
HSD	1081[c]	29·4[a]	9·0[a]
RSD	2538[b]	7·3[b]	6·3[b]
HSM	1247[c]	8·9[ab]	7·5[ab]
RSM	1595[c]	6·2[bc]	5·6[b]
HSD+BBTI	5233[a]	5·7[bc]	7·1[ab]
HSD+TOM	3156[b]	8·3[ab]	3·6[b]
Saline‡+BBTI	1760[c]	2·8[c]	2·7[b]
Saline	275[d]	8·0[b]	2·7[b]
SE of mean	400	1·4	0·30

[a, b, c, d] Values followed by a common superscript letter did not differ significantly ($P < 0.05$).

* For details of diets, HSM, RSM, BBTI and TOM, see pp. 238.

† One unit of activity was defined as a change in extinction of one extinction unit at 550 nm/3 min for amylase and at 410 nm/60 min for trypsin and chymotrypsin.

‡ 9 g sodium chloride/l.

in the caeca of chicks which received BBTI dissolved in saline as compared with those receiving saline only. However, amylase activity in the caecum was significantly higher when BBTI was mixed with food than when dissolved in saline. Amylase activity in the small intestine was not affected significantly by the ingestion of RSD or HSD supplemented with trypsin inhibitors. When saline or BBTI+saline was introduced to the crop, intestinal activity of amylase was lower than that with all other treatments (Table 3).

Trypsin and chymotrypsin in the small intestine were inhibited whenever RSD, RSM, BBTI or TOM was given to the chicks. In the caecum a similar trend was found although the differences were not always significant. Enzyme activities in the pancreas were not affected by the different treatments.

Expt 4. When one caecum was ligated, higher amylase activity was found in the small intestine and the non-ligated caecum after ingestion of RSD as compared with HSD, while amylase activities in the ligated caecum were similar in both groups. In the control, non-operated chicks, increased amylase activity after ingestion of RSD was evident only in the caecum (Table 4). Injection of BBTI directly into a ligated caecum did not affect amylase activity as compared with the caecum injected with saline.

Table 4. *Expt* 4. *Enzyme activities (units‡/g chyme) in the small intestine and caecum of chicks given one meal of heated (HSD)- or raw (RSD)-soya-bean diet* after ligation of one caecum or at the end of the ileum, or injection of trypsin inhibitor (BBTI) into a ligated caecum*†

(Mean values for six chicks/group)

	Amylase (*EC* 3.2.1.1)			Trypsin (*EC* 3.4.4.4)			Chymotrypsin (*EC* 3.4.4.5)		
	HSD	RSD	SE	HSD	RSD	SE	HSD	RSD	SE
Non-operated:									
Small intestine	1218[a]	1792[a]	175	28·5[a]	1·2[b]	1·5	10·0[a]	1·3[b]	1·2
Caecum	829[b]	3870[a]	214	16·4[a]	4·0[b]	3·0	4·2[a]	3·0[a]	0·9
Ligation of one caecum:									
Small intestine	529[b]	1390[a]	105	24·7[a]	2·5[b]	1·6	7·6[a]	1·2[b]	1·1
Ligated caecum	63[a]	94[a]	12	7·2[a]	11·4[a]	2·9	3·9[a]	2·9[a]	0·6
Non-ligated caecum	245[b]	2200[a]	159	15·9[a]	4·5[b]	2·3	4·2[a]	3·4[a]	0·8
Ligation at end of ileum:									
Small intestine	544[a]	533[a]	75	18·2[a]	2·2[b]	2·5	4·3[a]	1·0[b]	0·5
Caecum	42[a]	61[a]	13	—	—	—	12·0[a]	9·5[a]	1·2
Ligation of one caecum:	Saline‖	Saline+BBTI							
(direct injection)	477[a]	467[a]	60	—	—	—	—	—	—

[a, b] Values followed by a common superscript letter did not differ singificantly ($P < 0.05$). Statistical analysis was carried out separately for each enzyme in each segment.

* For details of diets, see p. 235.

† For details of procedure, see p. 238.

‡ One unit of activity was defined as a change in extinction of one extinction unit at 550 nm/3 min for amylase and at 410 nm/60 min for trypsin and chymotrypsin.

‖ 9 g sodium chloride/l.

Table 5. *Expt* 4. *Amylase and trypsin activities (units‡/g) in the pancreas and along the intestinal tract of chicks with ligated duodenum* 2 h *after introduction of heated soya-bean diet* (HSD) or HSD+trypsin inhibitor (BBTI) into the jejunum*†

(Mean values for six chicks/treatment)

	HSD	HSD+BBTI	SE of mean
	Amylase (*EC* 3.2.1.1)		
Pancreas	38040[a]	30995[a]	1553
Duodenum	62·0[a]	109[a]	35·8
Small intestine	20·2[a]	16·7[a]	6·9
Caecum	205[a]	76·9[a]	58·2
	Trypsin (*EC* 3.4.4.4)		
Pancreas	416[a]	360[a]	22·1
Duodenum	6·1[a]	6·0[a]	0·8
Small intestine	28·5[a]	0·87[b]	2·5
Caecum	13·3[a]	8·4[b]	1·2

[a, b] Values followed by a common superscript letter did not differ significantly ($P < 0.05$).

* For details of diets, see p. 235.

† For details of procedures, see p. 240.

‡ One unit of activity was defined as a change in extinction of one extinction unit at 550 nm/3 min for amylase and at 410 nm/60 min for trypsin.

Trypsin and chymotrypsin activities in the small intestines of all chicks were reduced after ingestion of RSD, as was found also in Expts 3 and 4 (Tables 3 and 4).

Trypsin inhibition was found also in the non-ligated caecum as well as in the caecum of non-operated chicks given RSD, while chymotrypsin-reduced activity in the caecum was not statistically significant. In the ligated caecum all enzyme activities measured were similar in the chicks given HSD or RSD.

When the end of the ileum was ligated, no increase in amylase activity was evident in the small intestine or caecum as a result of RSD ingestion. Trypsin and chymotrypsin were inactivated in the small intestine (Table 4).

Ligation at the end of the duodenum and injection of suspension of HSD or HSD + BBTI into the jejunum did not affect amylase levels in the pancreas or in any of the segments of the intestinal tract (Table 5). Trypsin activity was reduced in the small intestine and caecum of chicks injected with HSD + BBTI (Table 5). In the duodenum most of the trypsin was in the inactive form and therefore did not produce the enzyme-inhibitor complex.

DISCUSSION

Low levels of amylase were found in the anterior parts of the digestive tract of chicks (Table 1). Amylase found in the contents of the crop was probably mostly of food origin as amylase secretion in the crop of chicks is low, if any (Hill, 1971). A considerable part of this amylase was inactivated while passing through the glandular stomach and gizzard (Table 1), probably due to the low pH in these organs (Hill, 1971; Table 2). It seems, therefore, that most of the amylase found in the digestive tract posterior to the stomachs is of endogenous origin. Chicks given RSD or HSD supplemented with trypsin inhibitors (BBTI or TOM), as compared with HSD, had significantly higher amylase activity in the ileum and caecum, as found in the present (Table 1) and previous studies (Lepkovsky *et al.* 1966; Nitsan & Gertler, 1972). In the pancreas and other segments of the intestinal tract, this effect was much smaller and inconsistent (Lepkovsky *et al.* 1966; Gertler & Nitsan, 1970; Nitsan & Bruckental, 1977).

This phenomenon led to the following questions: whether the additional activity found in the caecum as a response to RSD ingestion was of pancreatic origin; or whether amylase was synthesized in the lower part of the intestinal tract by its mucosal cells or by the microflora as a response to RSD or trypsin inhibitors. The fact that in depancreatized chicken the decrease in starch digestion was only 30%, compared with a reduction of 75% in protein and fat digestion (Ariyoshi, Koike, Furuta, Ozone, Matsumura, Dimick, Hunterg, Wang & Lepkovsky, 1964), could mean that there might be some other sources of amylase, besides the pancreas, in the digestive tract of the chicks.

In a series of experiments in which ligations were made at different sites along the digestive tract, it was shown that ingestion of RSD was followed by an increase in amylase activity in the caecum only when the flow of intestinal contents could reach the caeca. The presence of trypsin inhibitors in the intestinal tract of chicks in which the caeca were ligated, did not induce any indirect stimulation (humoral factors) of caecal cells to secrete amylase. Direct stimulation, by injecting trypsin inhibitors into the caecum, also did not cause any increase in amylase level. It seems, therefore, that amylase was not synthesized in the caecum (either by caecal cells or by its microflora) as a response to the presence of RSD or trypsin inhibitors.

The same was true for the small intestine cells or microflora, as amylase levels were not affected by introducing trypsin inhibitors into the jejunum when the flow of pancreatic secretion into the intestine was prevented (ligation at the end of the duodenum, Table 4). Therefore, the high levels of amylase found in the caeca after ingestion of RSD or trypsin inhibitors probably originated from the pancreas.

A negative feedback mechanism, which was suggested to control pancreatic enzyme secretion in rats (Green & Lyman, 1972), might be involved also in chicks. Schneeman, Chang, Smith & Lyman (1977) suggested that the presence of trypsin and chymotrypsin in the upper one-third of the small intestine suppressed enzyme secretion, while their removal increased their secretion by rat pancreas. This type of mechanism seems to be involved in pancreatic secretion of amylase in chicks.

When the small intestine was ligated at its posterior end (Table 4), the movement of its chyme ceased and enzymes could not pass from the jejunum into the ileum or into either of the caeca. In this instance amylase activity level after ingestion of RSD did not increase, probably as a result of feedback control of pancreatic secretion. However, ingestion of RSD by chicks in which only one caecum was ligated and the intestinal chyme could move, resulted in the increase in amylase levels in both the free caecum and the small intestine. Pancreatic secretion of amylase increased and part of the amylase, which would normally drain into the second caecum, was probably retained in the lower part of the small intestine. Increase in amylase level at this site did not affect pancreatic secretion (Schneeman *et al.* 1977).

The upper part of the jejunum is believed to be the site which is involved in humoral pancreatic stimulation of enzyme secretion (Twombly-Snook, 1969; Green & Lyman, 1972; Schneeman & Lyman, 1975). Injection of HSD or HSD + BBTI into this site in chicks in which the end of the duodenum was ligated did not affect pancreatic enzyme levels (Table 5) as the enzymes could not be released from the pancreas.

Trypsin was inactivated whenever RSD or trypsin inhibitors were administered by crop intubation or by direct injection to any of the intestinal segments. Chymotrypsin inactivation was less consistent, as RSD contains much less chymotrypsin than trypsin inhibitors (Kakade, Simons, Liener & Lambert, 1972).

Diets containing trypsin inhibitors enhanced the secretory activity of the pancreas more than ingestion of RSM (Table 3) or trypsin inhibitors dissolved in saline, which agrees with the observation of Niess, Ivy & Nesheim (1972). It might be related to the rate of food passage through the digestive tract which was probably slower than for any of the soya-bean meals or saline containing the inhibitor, having, therefore, more a prolonged effect on the site stimulating pancreatic secretion.

The authors gratefully acknowledge the technical assistance of Mrs Y. Hass and Mrs V. Barak.

REFERENCES

Ariyoshi, S., Koike, T., Furuta, F., Ozone, K., Matsumura, Y., Dimick, M. K., Hunterg, W. L., Wang, W. & Lepkovsky, S. (1964). *Poult. Sci.* **43**, 232.
Bernfeld, P. (1955). *Meth. Enzym.* I, 149.
Birk, Y. & Gertler, A. (1968). *Biochem. Prep.* **12**, 25.
Erlanger, B. F., Edel, F. & Cooper, A. G. (1966). *Archs Biochem. Biophys.* **115**, 206.
Erlanger, B. F., Kokowsky, N. & Cohen, W. (1961). *Archs Biochem. Biophys.* **95**, 271.
Feeney, R. E., Osuga, B. T. & Maeda, M. (1967). *Archs Biochem. Biophys.* **119**, 174.
Gertler, A. & Nitsan, Z. (1970). *Br. J. Nutr.* **24**, 893.
Green, G. M. & Lyman, R. C. (1972). *Proc. Soc. exp. Biol. Med.* **142**, 1162.
Hill, K. J. (1971). In *Physiology and Biochemistry of the Domestic Fowl*, p. 25 [D. J. Bell and B. M. Freeman, editors]. London and New York: Academic Press.
Kakade, M. L., Simons, N. R., Liener, I. E. & Lambert, J. W. (1972). *J. Agric. Fd Chem.* **20**, 87.
Kayambashi, H. & Lyman, R. C. (1966). *J. Nutr.* **89**, 455.
Lepkovsky, S., Koike, T., Sugiura, M., Dimick, M. K. & Furuta, F. (1966). *Br. J. Nutr.* **20**, 421.
Liener, I. E. & Kakade, M. E. (1969). In *Toxic Constituents of Plant Foodstuffs*, p. 7 [I. E. Liener, editor]. New York: Academic Press.
Niess, E., Ivy, C. A. & Nesheim, M. C. (1972). *Proc. Soc. exp. Biol. Med.* **140**, 291.

Nir, I., Shapira, N., Nitsan, Z. & Dror, Y. (1974). *Br. J. Nutr.* **32**, 279.
Nitsan, Z. & Bruckental, I. (1977). *J. Anim. Sci.* **44**, 998.
Nitsan, Z. & Gertler, A. (1972). *Nutr. Metab.* **14**, 371.
Nitsan, Z. & Liener, I. E. (1976). *J. Nutr.* **106**, 300.
Schneeman, B. O., Chang, I., Smith, L. B. & Lyman, R. L. (1977). *J. Nutr.* **107**, 281.
Schneeman, B. O. & Lyman, R. L. (1975). *Proc. Soc. exp. Biol. Med.* **148**, 897.
Snedecor, G. W. (1962). *Statistical Methods*, 5th ed. Ames, Iowa: The Iowa State College Press.
Twombly-Snook, J. (1969). *J. Nutr.* **97**, 286.

Printed in Great Britain